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Human Papillomavirus Type 16 Sequence Variants: Identification by E6 and L1 Lineage-Specific Hybridization

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A catalog of human papillomavirus (HPV) type 16 (HPV-16) E6 and L1 signature nucleotides was used to develop PCR-based oligonucleotide probe systems capable of distinguishing HPV-16 class and subclass variants. Twenty-three E6-specific oligonucleotide probes targeting 13 variant nucleotide positions and 12 L1-specific oligonucleotide probes targeting 6 variant nucleotide positions were used to characterize HPV-16-containing cervicovaginal lavage specimens. Nucleotide positions that could be distinguished included E6 nucleotides 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532 and L1 nucleotides 6695, 6721, 6803, 6854, 6862, and 6994. Combined hybridization patterns were assigned on the basis of the predicted HPV-16 class, subclass, or minor class variants described previously (T. Yamada, C. M. Wheeler, A. L. Halpern, A.-C. M. Stewart, A. Hildesheim, and S. A. Jenison, J. Virol. 69:7743–7753, 1995). The major HPV-16 variant lineages detected included European prototype-like (E-P), Asian (As), Asian-American (AA), and African (Af1 and Af2) lineages. In addition, E-G131, an E-class variant, and AA-G183, an AA-class variant, were also identified. For each clinical specimen, DNA hybridization results were compared to nucleotide sequence determinations. Targeted L1 and E6 marker nucleotides covaried within all HPV-16 variant isolates examined. These hybridization-based methods result in minimal misclassification error, are amenable to targeting additional lineage-specific nucleotide positions, and should facilitate the large-scale, low-cost analysis of HPV-16 variants in epidemiologic investigations. Specifically, these methods will facilitate epidemiologic studies of HPV-16 transmission and natural history, as well as studies of associations between HPV variants, host immune responses, and cervical neoplasia.

Associations between human papillomavirus (HPV) and cervical neoplasia have been consistently reported (13, 33, 40, 41, 52). In longitudinal studies (23, 25), persistent repeated detection of cancer-associated HPVs is a strong risk factor for the development of cervical intraepithelial neoplasia, and high viral load is also presumably a contributing factor (24). HPV type 16 (HPV-16) is associated with the majority of cervical cancers worldwide (5), and thus provides the greatest opportunity to further consider biological modifiers of HPV-associated disease risk. Host immunologic factors appear to be important modifiers of risk; however, the role of sequence variation within HPVs as it may relate to host immune response, persistence, or risk for cervical intraepithelial neoplasia and invasive cancer has not been thoroughly examined.

HPV genotypes have been used as the exposure measure for HPV-associated risk for invasive cervical carcinoma. Currently, a papillomavirus (PV) genotype is defined as a viral DNA having less than 90% nucleotide similarity to any other PV genotype in the L1 open reading frame (ORF), a PV subtype is defined as a viral DNA having between 90 and 98% similarity, and a PV variant is defined as a viral DNA having more than 98% nucleotide similarity (11, 46, 63). PCR coupled to hybridization methods has been widely used to differentiate HPV genotypes (1, 31, 36, 50, 57, 60–62). However, the large number of disease-relevant HPV genotypes identified during the past decade has posed a challenge to the development of simplified HPV detection. Consequently, variants of HPV genotypes have been given little consideration.

In general, efforts to study PV variants have been based on direct determinations of DNA sequence and have concentrated on HPVs. Studies of sequence variation in epidermodysplasia verruciformis-related HPVs have focused on HPV-5 and HPV-8 (9, 10). For genital HPVs, studies of variants have been directed at establishing intra- and intertypic phylogenetic relationships, with sequence-specific information being primarily limited to studies of HPV-16 and HPV-18 (9, 12, 14, 16, 17, 20, 21, 26–30, 45, 48, 49, 54, 56, 58, 70). A few of these studies have examined sequence-specific changes and disease associations but were limited to the E2, E6, E7, and/or L1 ORFs (12, 17, 20, 30, 49, 56). In addition, the identification of specific HPV variants in sex partners has been used to consider HPV transmission dynamics (26), and studies of HPV persistence have used variant analysis to examine persistent versus newly acquired HPV infections (15, 67). Recent data provides support for the functional significance of HPV-16 sequence variants; both virus-like particle assembly (32) and altered immortalization properties (8) have been observed.

In the past few years, virus-like particles comprised of the HPV major capsid protein (L1) or the major and minor capsid proteins in combination (L1 and L2) have been under consid-

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eration as HPV vaccine candidates (42). We have previously noted several HPV-16 class-specific amino acid changes in the E6-, L1-, and L2-coding regions (70). These amino acid sequence variations may be relevant to the generation of protective immune responses against HPV-16 including virus neutralization. Similarly, amino acid variation within the E6-coding region may be relevant to differences in oncogenic potential. It seems reasonable to investigate the prevalence of HPV-16 variants in any population under consideration for HPV-16-based immunization.

To facilitate the further consideration of HPV-16 sequence variation in the rational design of vaccine strategies and as an epidemiologic correlate of HPV-16-associated disease, we have developed simplified hybridization-based methods for detecting the previously described major HPV-16 lineages (i.e., European [E], Asian [As], Asian-American [AA], and African [Af1 and Af2]) (26, 27, 70). In addition to these major HPV-16 variant lineages, our methods distinguish two previously reported lineage subclasses including G131, an E-class variant, and G183, an AA-class variant (70). We have concentrated our efforts on the widely targeted MY09/11 region of the L1 ORF (38) and the E6-coding segment.

Twenty-three E6-specific oligonucleotide probes targeting 13 variant nucleotide positions and 12 L1-specific oligonucleotide probes targeting 6 variant nucleotide positions were used to characterize HPV-16-containing clinical specimens previously identified during a natural history study of cervical disease in Portland, Oreg. (52). Probes were designed to detect the HPV-16 E6 and L1 variant sequences present in previously described variant lineages (70) as well as the prototypic or reference sequence at each nucleotide position.

To examine the general utility of these HPV-16 lineage-specific probe-based systems, we compared the hybridization results obtained during routine dot blot analysis to actual DNA sequence information.

MATERIALS AND METHODS

Preparation of clinical specimens. HPV-16 DNA-containing cervicovaginal lavage samples were obtained from subjects enrolled in a natural history study of HPV-associated cervical disease conducted at the Kaiser Permanente health maintenance organization, Portland, Oreg. (52). Specimens included a range of cytologic diagnoses and were predominantly normal. Two hundred microliters of 2× digestion solution containing 400 μg of proteinase K per ml and 2% (vol/vol) Laureth-12 prepared in 50 mM Tris-HCl (pH 8.5)–1 mM EDTA was added to an equal volume of resuspended cervicovaginal lavage specimen. Digestion was carried out for 1 h at 55°C, followed by inactivation of the proteinase at 95°C for 10 min.

PCR. *Thermus aquaticus* (*Taq*)-based PCRs were performed as 100-μl amplification reactions containing 10 mM Tris (pH 8.5), 50 mM KCl, 200 μM (each) deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 or 4.0 mM $MgCl_2$, 0.125 to 0.5 μM (each) sense strand and antisense strand oligonucleotide primers, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Foster City, Calif.). Sequential two-tube nested PCRs were performed in order to increase the yields of the desired amplicons in both the E6- and L1-based systems. Two microliters of crude DNA from the clinical specimens was used in the initial amplification reaction mixtures. Two microliters of the resultant amplification product was then added to the second nested amplification reaction mixture.

All PCR tubes were opened with sterile gauze squares, and gloves were changed after testing every 12 specimens. Potential contamination was controlled for by interspersing HPV-negative and no-DNA control reaction mixtures. In addition, amplification reactions were conducted independently for the hybridization experiments and the DNA sequence analyses. To eliminate PCR product contamination, a one-tube nested PCR method of equivalent sensitivity (i.e., both assays detect a theoretical single-copy genome input as determined by limiting dilution experiments) was developed for the E6-coding region and was applied in subsequent studies (64, 69). Although not used for the studies described in this report, we have included the details for this amplification system because it is our intention that this report serve as a reference for the performance of these methods. For this one-tube nested system two primer pairs were added to the initial amplification reaction mixture. Nested amplification was achieved by designing an outer primer pair with a predicted annealing temperature of 57°C and an inner primer pair with a predicted annealing temperature of 45°C; the outer primer pair concentration was limiting at 0.1 μM. Amplification was conducted in a single tube by using 30 cycles with an annealing temperature of 57°C, followed by a second amplification round of 35 cycles with an annealing temperature of 45°C. PCR conditions and oligonucleotide primer sequences for all PCR amplification systems are presented in Table 1.

Amplification reaction mixtures that were intended for subsequent direct DNA sequence analysis included a single biotinylated primer, which is designated with an asterisk in Table 1. Both biotinylated sense and antisense primers were used in the internal nested L1 amplification. We did not use a biotinylated primer in reaction mixtures that were targeted for subsequent analysis by dot blot hybridization, because biotinylated oligonucleotide probes were applied in the subsequent detection of variant sequences by hybridization. All resultant amplification products were evaluated by agarose gel electrophoresis in 0.5× Tris-borate-EDTA running buffer. Gels were stained with ethidium bromide (EtBr), and DNA was visualized by UV transillumination.

Dot blot hybridization. Sixty microliters of each amplified sample was mixed with 1 ml of 0.4 M NaOH and 25 mM EDTA. After 10 min of denaturation at room temperature, 50 μl (per well) was then applied to a Biodyne-B nylon membrane (ICM Biochemicals, Irvine, Calif.) with a 96-well dot blot manifold. In effect, this results in the application of 3 μl of the original amplified sample to each well. Following the complete aspiration of the denatured samples under vacuum, each well was rinsed with 400 μl of 20× SSPE (3.6 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.11 M NaOH, and 0.02 M disodium EDTA $\cdot 2H_2O$ adjusted to pH 7.4), and the vacuum was reapplied until the wash solution was completely removed. Nylon membranes were removed from the manifold under complete vacuum, and the bound DNA was immediately cross-linked to the membranes with a Stratalinker UV cross-linker on the autolink setting (Stratagene, San Diego, Calif.). Membranes were then stored in 2× SSPE at room temperature until subsequent hybridization.

The biotinylated oligonucleotide probes listed in Table 2 were designed by using control HPV-16 DNAs identified in our previous investigations (70). The nucleotide variations targeted by these probes included HPV-16 E6 nucleotide positions 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532 and L1 nucleotide positions 6695, 6721, 6803, 6854, 6862, and 6694.

Immediately prior to hybridization with HPV-16 lineage-specific oligonucleotide probes, membranes were pretreated at 65°C in 0.1× SSPE containing 0.5% sodium dodecyl sulfate (SDS) under vigorous shaking conditions. No more than six membranes per 1.5 liters of pretreatment solution were used. Hybridizations with biotinylated probes (1.0 pmol of probe per 50 ml of hybridization solution) were conducted for a minimum of 2 h or overnight in 5× SSPE containing 0.1% SDS. At least 100 ml of hybridization solution was used for up to six membranes. Hybridizations for all E6 and L1 biotinylated probes listed in Table 2 were conducted at 42.5°C. Following the incubation at 42.5°C, the hybridization solution was drained from the membranes, which were then rinsed at room temperature in 1 liter of 2× SSPE containing 0.1% SDS. Membranes were subsequently subjected to two consecutive 10-min washes with 2 liters of 2× SSPE that had been prewarmed to the desired wash temperature. All membranes were washed at 44.0°C except for those hybridized with E6 131/2AT, E6 131/2AG, E6 350T, and E6 403G probes. Washes were conducted for these probes at 47.0°C. Membrane washes were followed by a final wash for 10 min at room temperature in the same solution.

By using a dedicated plastic tray, strepavidin (SR)-horseradish peroxidase (HRP) conjugate (1 mg/ml; Vector Laboratories, Burlingame, Calif.) was prepared to a final concentration of 30 ng per ml (7.5 μl per 250 ml) in 2× SSPE containing 0.1% SDS. Binding of the SA-HRP was allowed to occur under gentle agitation for 20 min at room temperature. Longer incubation periods ultimately resulted in higher levels of background signals. The SA-HRP solution was drained from the membranes, which were then briefly rinsed in 2× SSPE containing 0.1% SDS at room temperature. The membranes were washed twice with 2 liters of this same solution under vigorous mixing for 10 min at room temperature.

Individual membranes were incubated for 1 min in equal volumes of A and B enhanced chemiluminescent substrate reagents (ECL; Amersham, Arlington Heights, Ill.) essentially as described by the manufacturer. The membranes were drained on filter paper and were immediately covered with plastic wrap for subsequent exposure to Kodak X-OMAT AR-5 film. Exposures were conducted generally for 2 to 10 min and were followed immediately by a second 2-h exposure. The exposure times were sometimes manipulated to obtain optimal signal intensities, as judged by evaluation of the hybridization controls present on each membrane. Controls were prepared by subjecting previously characterized clinical specimens (70) to amplification in the E6 and L1 PCR systems listed in Table 1. At least two representative controls from HPV-16 class and subclass variants including E, E-G131, As, AA, AA-G183, Af1, and Af2 were included on each membrane for both the E6 and L1 hybridization assays. In addition, a European variant designated C109 was also included. DNA sequence information had previously been obtained for the complete E6-, L2-, L1-, and long control region (LCR)-coding segments in all control specimens (70).

Hybridization data analysis. E6 and L1 hybridization assay results were interpreted from the chemiluminescent signals recorded on X-ray film. The results were entered into a database file (Dbase III program; Borland International, Inc., Scotts Valley, Calif.) containing fields for all probes under study. Data entry was manually performed without knowledge of the corresponding DNA se-

TABLE 1. HPV-16 E6 and L1 Nested PCR amplification systems[a]

Primer position		PCR type	Sense and antisense strand primers[b]	Primer concn (μM)	PCR conditions[c]				
5′	3′				No. of cycles	Temp (°C), time (s)			
						D	A	E	TE
31	683	Two-tube E6 nested PCR (outer reaction)[d]	S: CGTAACCGAAATCGGTTGAAC	10.0	35	94, 30	52, 30	72, 60	72, 300
			A: GGACCATCTATTTCATCCTCCT	10.0					
31	640	Two-tube E6 nested PCR (inner reaction)[d]	S: CGTAACCGAAATCGGTTGAAC	20.0	25	94, 30	55, 30	72, 60	72, 300
			A: *GCTCATAACAGTAGAGATC	20.0					
31	683	One-tube E6 nested PCR (outer reaction)[d]	S: CGTAACCGAAATCGGTTGAAC	0.10	30	94, 30	57, 30	72, 60	72, 300
			A: GGACCATCTATTTCATCCTCCT	0.10					
56	640	One-tube E6 nested PCR (inner reaction)	S: ACCGGTTAGTATAAAAG	20.0	35	94, 30	45, 30	72, 60	72, 300
			A: *GCTCATAACAGTAGAG	20.0					
6584	7035	Two-tube L1 nested PCR (outer reaction, MY09/11[e])	S: GCMCAGGGWCATAAYAATGG	50.0	35	94, 30	55, 30	72, 60	72, 300
			A: CGTCCMARRGGAWACTGATC	50.0					
6599	7021	Two-tube L1 nested PCR (inner reaction)	S1: *AATGGCATTTGTTGGGGTA	20.0	25	94, 30	52, 30	72, 60	72, 300
6598	7021		S2: *TAATGGCATTTGTTGGGGT	20.0					
			A: *CTGATCTAGGTCTGCAGAAAAC	20.0					

[a] All PCR amplifications except the MY09/11 reaction were conducted with a final concentration of 2.5 mM Mg^{2+}; the MY09/11 reaction was conducted with 4.0 mM Mg^{2+}.

[b] Oligonucleotide probe sequences are written in the 5′ to 3′ direction. Positions at which more than one nucleotide was incorporated during the DNA synthesis are indicated as follows: M = A + C, R = A + G, W = A + T, Y = C + T. S, sense; A, antisense. *, a 5′-biotinylated primer that was sometimes used during amplification to facilitate subsequent DNA sequencing efforts.

[c] PCR conditions include the number of amplification cycles, and the temperature and time of the denaturation (D), annealing (A), extension (E), and terminal extension (TE) steps.

[d] The PCR was reported previously (70).

[e] The PCR was reported previously (37).

TABLE 2. HPV-16 E6 and L1 variant-specific oligonucleotide probes

Nucleotide position[a]	E6 probe sequence[b]	Nucleotide position	E6 probe sequence	Nucleotide position	MY09/11 L1 probe sequence
109T	CAATGTTTCAGGACC	183T	CATGATATAATATTAGAATG	6695A	CTTCAGAAACTACATAT
109C	CAATGTTCCAGGACC	183G	ATGATATAAGATTAGAATGT	6695C	CTTCAGAACCTACATAT
131/2AG*	CGACCCAGAAAGTTAC	286/9TA	CATATGCTGTATGTGAT	6721A	AACTTTAAAGAGTACCTA
131/2GG	ACCCGGAAAGTTACC	286/9AG	ATATGCAGTGTGTGATA	6721G	CTAACTTTAAGGAGTAC
131/2AC	GACCCACAAAGTTAC	335C	GTATAGACATTATTGTTAT	6803A	CGTTATGACATACATAC
131/2AT*	GACCCATAAAGTTACC	335T	GTATAGATATTATTGTTATA	6803T	CGTTATGTCATACATAC
143/5CG	GTTACCACAGTTATGC	350T*	TTGTTATAGTTTGTATGGAA	6854/62CT	TGGTCTACAACCTCC
143/5GT	GTTACCAGATTTATGCA	350G	TTGTTATAGTGTGTATGGAA	6854/62CC	TGGTCTACAACCCCC
143/5CT	TTACCACATTTATGCAC	403A	TGTGATTTGTTAATTAGGT	6854/62TT	TTGGTTTACAACCTCC
178T	CTATACATGATATAATATTAG	403G*	GTGATTTGTTGATTAGGT	6854/62TC	TTGGTTTACAACCCCC
178G	TATACATGAGATAATATTAG	532A	AGATCATCAAGAACACG	6994G	TAAAGGAAAAGTTTTCTG
		532G	GATCATCGAGAACAC	6994A	TTAAAAGAAAAGTTTTCTG

[a] Nucleotide positions and specific HPV-16 nucleotide variations targeted are shown to the left of each column. *, a wash temperature of 47°C for designated probes.

[b] Oligonucleotide probe sequences are written in the 5′ to 3′ direction, and targeted nucleotide positions are underlined. Probe hybridization and wash conditions are described in Materials and Methods.

quence data. The results for each oligonucleotide probe were coded as 0 (negative), 1 (strong positive), or 2 (weak positive). An analysis program was written by using SAS (SAS Institute Inc., Cary, N.C.) to facilitate the linkage of individual oligonucleotide probe results. This program can be obtained from one of the authors (C. M. Wheeler). Linked results were used to establish an overall hybridization pattern for the targeted HPV-16 E6 and L1 regions in each specimen examined. Combined hybridization patterns were assigned on the basis of the predicted HPV-16 class, subclass, or minor class variants observed previously (70). These assignments were capable of distinguishing E-class variants including E-350T, E-350G, E-C109/350G, E-C109/350T, E-G131/350G, and E-G131/350T as well as As, AA, AA-G183, Af1, and Af2 variants. In addition, ambiguous or novel patterns could be distinguished. Characteristic or representative patterns for each of the HPV-16 variants observed in this study, including all ambiguous or novel patterns, are presented in Fig. 1 and 2.

Nucleotide sequence analysis. Direct nucleotide sequencing of biotinylated PCR amplicons was facilitated by the use of SR-coated magnetic beads (Dyna-

beads; Dynal, Lake Success, N.Y.). The nucleotide sequence was determined by the dideoxy termination method (51) with the Sequenase, version 2.0, kit (United States Biochemicals, Cleveland, Ohio) with HPV-16-specific sequencing primers. Nucleotide sequence data was obtained for the complete E6-coding region in 136 of 157 HPV-16 containing specimens examined. In addition, the nucleotide sequence was obtained for those specimens demonstrating ambiguous patterns in either the targeted E6 or L1 region.

HPV-16 nucleotide position numbering. HPV-16 DNA nucleotide positions are numbered according to the published sequence of the reference clone (53), but revised to include corrections reported by Bubb et al. (6), Halbert and Galloway (19), and Parton (47). As in our earlier work on HPV-16 variants (70), we have included an additional correction, replacing the published GC at position 7432 with CGG, communicated to us by Bernard (4) and observed in the LCR sequences of our HPV-16 isolates. In addition, we have included a G inserted at nucleotide 1139 in the E1 ORF (38). In comparison to our earlier report, this addition results in an increase of 1 nucleotide in the numbering within the L1 and LCR sequences presented here. This numbering scheme corresponds to the HPV-16R sequence presented in a recent compendium of HPV sequences (43).

LCR DNA sequence information was obtained for the purpose of correlating the designations in this work with previously published phylogenetic designations (26).

RESULTS

HPV-16 E6 hybridizations. One hundred sixty cervicovaginal lavage specimens were previously characterized by the MY09-11 L1 consensus PCR system (data not shown). One hundred fifty-three specimens that contained HPV-16 DNA, four that contained both HPV-16 and HPV-31 DNAs, and three in which only HPV-31 DNA was detected were evaluated in the E6 hybridization assays described below. All specimens were amplified in a two-tube nested E6 PCR detailed in Table 1. The resultant amplimers were examined by agarose gel electrophoresis, subjected to DNA sequence analysis, and hybridized with the 23 oligonucleotide probes listed in Table 2. These probes were designed to detect all class- and subclass-specific HPV-16 nucleotide variations identified in our earlier investigations (70). These earlier studies used more than 100 kb of DNA sequence information to establish phylogenetic relationships between HPV-16 variants. Many probes were designed and evaluated against HPV-16 variants of known sequence before this final battery was selected.

For purposes of explanation, we have previously designated the four major HPV-16 lineages E, AA, Af1, and Af2 as classes (70). Bootstrap values of 95% or more were obtained for each lineage. Lineages with bootstrap values of between 90 and 95% were designated as subclasses (70). Each pair or group of probes distinguished the reference HPV-16 sequence from any variant nucleotide position(s) previously noted. The control amplification reaction mixtures described above in Materials and Methods and clinical specimens that contained HPV type 33, 35, 52, and 58 DNAs were also amplified and subjected to E6 hybridization assays. These specimens were previously characterized by type-specific hybridization of MY09-11 L1 PCR products (1, 18, 23, 55) and complete sequencing of the MY09-11 L1 fragment (55). They were included during the hybridization assays to consider potential confounding of hybridization results by other prevalent group A HPVs (44).

Evaluation of the amplification reactions by agarose gel electrophoresis and EtBr staining revealed that all HPV-16 DNAs were amplified by the E6 two-tube nested reaction. When evaluated by agarose gel electrophoresis with EtBr staining, the nested PCR appeared to result in a maximal and equivalent production of PCR amplimers in greater than 95% of the specimens evaluated. This was achieved presumably because most amplification reactions had reached the plateau of achievable logarithmic amplification. Specimens containing HPV types 16 and 31, HPV-31 DNA alone, and HPV-58 were readily amplified by the nested amplification system. No bands of the appropriate molecular weight were seen with specimens containing HPV type 33, 35, or 52. Initial evaluation of the outer PCR amplification reaction by EtBr staining in agarose gels indicated that a nonnested amplification with these selected primers was insufficient to generate a visible PCR product in greater than 30% of the specimens targeted.

Representative E6 hybridization patterns including all ambiguous patterns obtained with the battery of 23 oligonucleotide probes are shown in Fig. 1 and 2. Expected signature nucleotides for each variant class and subclass (i.e., E-P, E-G131G, E-As, Af1, Af2, NA1, AA, and AA-G183G) can be distinguished in Fig. 1. Of the 160 specimens under evaluation, 156 specimens containing HPV-16 DNA hybridized well with the majority of the 23 probes applied. A single specimen containing both HPV-16 and HPV-31 DNAs failed to hybridize to any of the E6-specific probes. This was presumably due to technical error.

A total of 11 ambiguous E6 hybridization patterns were observed in specimens containing only HPV-16 DNA. Ambiguous probe positions shown in Fig. 2 were defined with the symbols described in the legend to Fig. 2. DNA sequence information from those specimens demonstrating ambiguous E6 hybridization patterns revealed eight ambiguities with single probes that were due to simple technical hybridization failures (asterisks for specimens OR.1664, OR.1790, OR.3604, OR.6152, OR.6282, OR.8685 and a question mark for OR.7145 in Fig. 2), two that were due to variant nucleotides occurring within the probe binding site (asterisks for specimens OR.0289 and OR.8005 in Fig. 2), and two that could be classified as having a mixed infection with two distinct HPV-16 variant classes (question mark or number sign for specimens OR.1783 and OR.4072 in Fig. 2). A single ambiguous overall E6 probe pattern was observed for specimen OR.1790, although two technical hybridization failures (131/2AG and 183G) were observed.

The distribution of technical hybridization failures was as follows: a single 109T probe (2.2% of the paired 109T and 109C probes); three 131/2AG probes, a single 131/2GG probe, and a single 131/2AT probe (<1% of the 131/2 probe group); a single 183T probe; and a single 143/5CG probe (1.4% of the 143/5 probe group). As shown in Fig. 2, nucleotide sequence variations differed from the anticipated HPV-16 E6 hybridization patterns in 2 (<2%) of the 157 specimens containing HPV-16 or HPV-16 plus HPV-31. In these cases nucleotide changes were identified by DNA sequence analysis at E6 nucleotide positions 135 and 187. These nucleotide variations were located in probe binding sites and provided explanations for two of the ambiguous E6 hybridization patterns. As mentioned previously, only a single specimen containing both HPV-16 and HPV-31 DNAs failed to hybridize with any of the 23 probes. Of 3,519 hybridizations to individual HPV-16-containing specimens (153 specimens with 23 probes), fewer than 0.01% technical hybridization failures were observed in the HPV-16 E6 probe system described.

Except for the two specimens containing a mixture of two HPV-16 variant classes, ambiguous probe patterns could be readily assigned to one of the previously described major classes of HPV-16 variants without subsequent DNA sequence analysis. A representative X-ray film exposure from the E6-based hybridizations targeting nucleotides 350G, 350T, 286/9TA, and 286/9AG is shown in Fig. 3.

HPV-16 L1 hybridizations. Our previous investigations indicated that the nucleotide changes observed in one region of the HPV-16 genome were linked to changes in other parts of the genome (70). In an attempt to further evaluate this observation, we developed a hybridization system that is capable of distinguishing six variant nucleotide positions within the MY09/11 L1 region, including nucleotides 6695, 6721, 6803,

ID	E6 PROBE RESULTS	E6 DNA SEQUENCE	MY09/11 PROBE RESULTS	MY09/11 DNA SEQUENCE	LCR DNA SEQUENCE	CLASS-SUBCLASS	n
			666666	666666666	77777777777777777		
	1111111223345	1111111222223345	678889	678888999	44556677777777788888		
	0334478883503	0334478566883503	920569	920566679	88026812345688233334		
	9123583695032	9123583679695032	513424	513425504	59719949032416647792		
REF.		TAGCGTTCGGTACTAA		AGACTCACG	AGAGCCTAATACTCGGAAG		
OR.5110	----------	----------	------	---------	----------	E-P	14
OR.4724	----------	----------	------	---------	---A------	E-P	1
OR.6170	C---------	C---------	------	---------	----------	E-P	1
OR.8329	C--------G-	C----------G--	------	---------	---A------	E-P	8
OR.6311	----------G-	-------T-----G--	------	---------	---A------	E-P	12
OR.4997	----------G-	---------AA---G--	------	---------	---A------	E-P	1
OR.9237	-G--------G--	-G----------G--	----C-	----C-C--	---A------	E-G131G	1
OR.0198	-G--------G--	-G----------G--	----C-	----C----	---A------	E-G131G	9
OR.7574	-----G-------	-----G---------	------	--------*	---A----C---C-----A	E-As	1
OR.5428	-----G-------	-----G---------	------	---------	---A----C---------A	E-As	3
OR.7587	--CGT--AGT---	--CGT-----AGT---	-A-T-A	-A-T---TA	-A-A-A-----T-T-T---	Af1	3
OR.1905	--CGT--ACT---	--CGT-----AGT---	-A-T-A	-A-T---TA	-A-A-AA----T-T-T -	Af1	1
OR.3473	C-TGT--AGT-G-	C-TGT-----AGT-G-	CA-T-A	CA-T-T-TA	CA-ATA-----T-TATCG-	Af2	3
OR.3136	----T--AGTG--	----T-----AGTG--	CA-T-A	CA-T-T-TA	CA-ATA-C---T-TAT---	NA1	1
OR.8160	----T--AGTG-G	----T-----AGTG-G	CATT-A	CATT-T-TA	CA-ATA-C-G-T-T-----	AA	5
OR.4541	----T--AGTG-G	----T-----AGTG-G	CATT-A	CATT-T-TA	CA-ATA-C-GCT-T-----	AA	1
OR.5691	----T-GAGTG-G	----T-G---AGTG-G	CA-TCA	CA-TCT-TA	CAGATA-C---T-T-----	AA-G183G	1
OR.8392	----T-GAGTG-G	----T-G---AGTG-G	C*-TC*	CA-TCT-TA	A-T---T-T-----	AA-G183G	1

REGIONS SEQUENCED

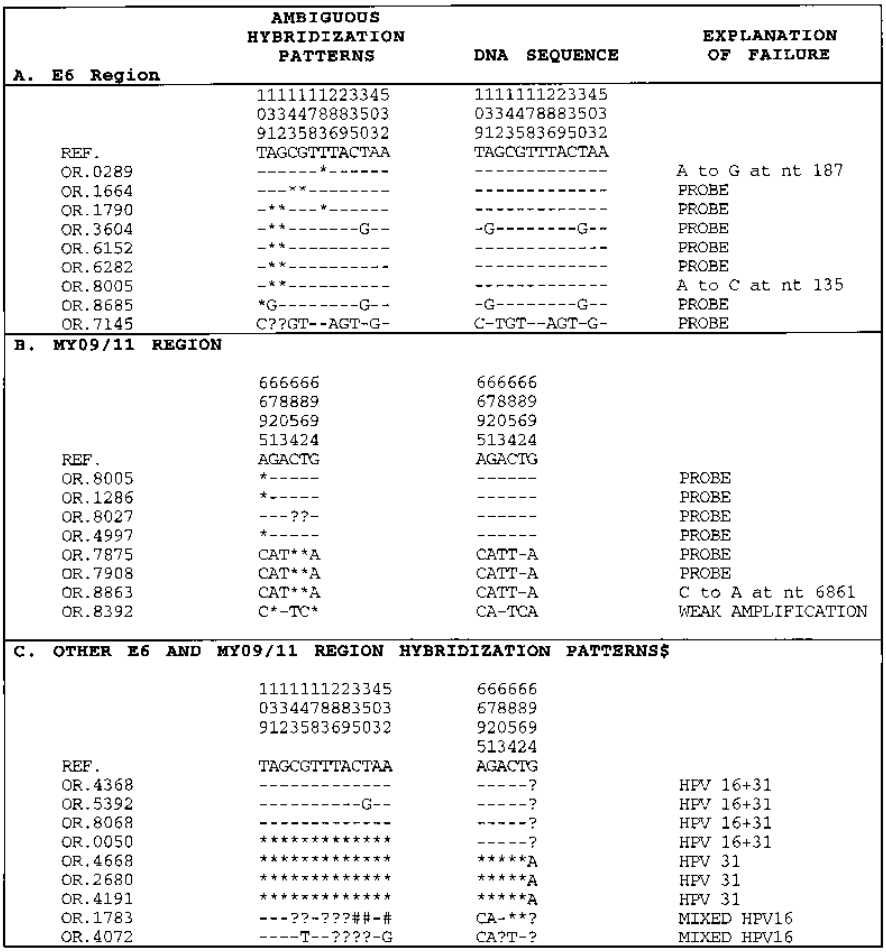
E6: nt 104-559 456bp
MY0911: nt 6618-6999 382bp
LCR: nt 7480-7843 364bp (nt7673-7843 for OR.8392)

FIG. 1. All nucleotide (nt) sequence variations among the HPV-16-containing clinical specimens. Both DNA sequence analysis and corresponding oligonucleotide probe hybridization data are provided for 67 specimens in which E6, partial L1, and LCR sequences were determined. Overall hybridization patterns were generated by computer-assigned nucleotide designations as described in Materials and Methods. The identification codes of the samples indicated along the left correspond to specimens obtained from Portland, Oreg. (52). Nucleotide sequences for E6, L1, L2, and LCR have been reported previously for these specimens (70). The corrected HPV-16 DNA reference sequence HPV-16R (43) is indicated as REF. For each variant sequence, positions that do not vary relative to the HPV reference sequence are marked with dashes. Phylogenetic groupings are indicated along the right, as are the numbers identified within each grouping. *, a negative hybridization result for the paired probes targeting nucleotide positions 6721 and 6994 in specimen OR.8392.

6854, 6862, and 6694. For the purposes of these studies, nucleotide changes in the widely targeted MY09/11 L1 region that were linked to changes in the E6 region were considered. These nucleotide variations are capable of distinguishing five of the main HPV 16 classes or subclasses including E, E-G131, AA, Af1, and Af2. A battery of 12 probes was designed to detect both the reference and the variant nucleotide sequences observed in our previous studies (70). The two-tube nested PCR detailed in Table 2 was applied to 70 of the HPV-16-containing specimens. HPV-16 E6 variants identified by DNA sequence analysis as well as a representative sampling of the 153 HPV-16-containing specimens evaluated in the E6 studies were included. Four specimens containing both HPV-16 and HPV-31 DNAs, three specimens containing HPV-31 DNA alone, and two specimens containing a mixed infection with two distinct HPV-16 variant classes were also included. In addition to the control amplification reactions described above in Materials and Methods, clinical specimens that contained HPV type 18, 33, 35, 45, 52, and 58 DNAs were also amplified and subjected to HPV-16 L1-specific hybridization assays. The complete nucleotide sequence of the MY09/11 L1 fragment in all control specimens had been determined previously (55). As in the E6 studies, specimens containing other HPV types were included during the L1-specific hybridization assays to consider potential confounding of the hybridization results by prevalent HPV types.

Evaluation of HPV-16 L1 amplification reactions by agarose gel electrophoresis and EtBr staining revealed that all HPV-16-containing specimens selected were amplified in the L1 two-tube nested reaction. Similar to the E6 nested amplification system, an apparent maximal and equivalent production of PCR amplimers was observed in greater than 95% of the specimens. Without nesting, resultant amplimers were extremely variable in terms of the band intensities observed. All specimens containing other HPV types including HPV types 18, 31, 33, 35, 52, 58, 18, and 45 were amplified in this two-tube nested system. When reconsidering the design of the inner nested primer pair, we noted that we could most likely eliminate the amplification of several of these HPVs by removing the 3′ nucleotide from the inner sense primer. Subsequent to these studies, a 5′ internal primer was developed by shifting the overall primer sequence a single nucleotide to the left. The sequence of this new primer beginning at nucleotide position 6598 is as follows: 5′-TAATGGCATTTGTTGGGGT-3′. This primer now replaces the sense primer of the inner reaction for the two-tube nested L1 amplification system and has been used successfully in our subsequent studies (64, 69).

Ambiguous probe positions were defined as described above for the E6 hybridizations (the symbols described in the legend to Fig. 2). Representative L1 hybridization patterns including all ambiguous patterns obtained with the battery of 12 L1-specific oligonucleotide probes are shown in Fig. 1 and 2. The HPV-16-containing specimens hybridized with the majority of the 12 L1-specific probes applied. A total of nine ambiguous patterns were observed in the specimens containing HPV-16 DNA alone. DNA sequence information from those specimens demonstrating ambiguous L1 hybridization patterns revealed six ambiguities with single probes that were due to technical hybridization failures (asterisks for specimens OR.8005, OR.1286, OR.4997, OR.7875, OR.7908 and a question mark for OR.8027 in Fig. 2), one that was due to a variant nucleotide occurring within the probe binding site (asterisk for specimen OR.8863 in Fig. 2), and two that were classified as having a mixed infection with two distinct HPV-16 variant classes (question mark or asterisk for specimens OR.1783 and OR.4072 in



A. E6 Region	AMBIGUOUS HYBRIDIZATION PATTERNS	DNA SEQUENCE	EXPLANATION OF FAILURE
	1111111223345	1111111223345	
	0334478883503	0334478883503	
	9123583695032	9123583695032	
REF.	TAGCGTTTACTAA	TAGCGTTTACTAA	
OR.0289	------*------	----------	A to G at nt 187
OR.1664	---**--------	----------	PROBE
OR.1790	-**---*------	----------	PROBE
OR.3604	-**-------G--	-G--------G--	PROBE
OR.6152	-**----------	----------	PROBE
OR.6282	-**----------	----------	PROBE
OR.8005	-**----------	----------	A to C at nt 135
OR.8685	*G--------G--	-G--------G--	PROBE
OR.7145	C??GT--AGT-G-	C-TGT--AGT-G-	PROBE
B. MY09/11 REGION			
	666666	666666	
	678889	678889	
	920569	920569	
	513424	513424	
REF.	AGACTG	AGACTG	
OR.8005	*-----	------	PROBE
OR.1286	*-----	------	PROBE
OR.8027	---??-	------	PROBE
OR.4997	*-----	------	PROBE
OR.7875	CAT**A	CATT-A	PROBE
OR.7908	CAT**A	CATT-A	PROBE
OR.8863	CAT**A	CATT-A	C to A at nt 6861
OR.8392	C*-TC*	CA-TCA	WEAK AMPLIFICATION
C. OTHER E6 AND MY09/11 REGION HYBRIDIZATION PATTERNS$			
	1111111223345	666666	
	0334478883503	678889	
	9123583695032	920569	
		513424	
REF.	TAGCGTTTACTAA	AGACTG	
OR.4368	----------	-----?	HPV 16+31
OR.5392	----------G--	-----?	HPV 16+31
OR.8068	----------	-----?	HPV 16+31
OR.0050	**********	-----?	HPV 16+31
OR.4668	**********	*****A	HPV 31
OR.2680	**********	*****A	HPV 31
OR.4191	**********	*****A	HPV 31
OR.1783	---??-???#-#	CA-**?	MIXED HPV16
OR.4072	----T--????-G	CA?T-?	MIXED HPV16

FIG. 2. All ambiguous HPV-16 hybridization patterns observed. DNA sequences were obtained for HPV-16 isolates demonstrating ambiguous patterns. The corrected HPV-16 DNA reference sequence HPV-16R (43) is indicated as REF. For each variant sequence, positions that do not vary relative to the HPV reference sequence are marked with dashes. Explanations for ambiguous results are indicated on the right. The word PROBE indicates that the result was due to a technical hybridization failure. When specific nucleotide (nt) changes were identified within the probe binding site, the nucleotide change is designated. Additional reasons for ambiguous patterns included weak PCR amplification, non-HPV-16 (i.e., HPV-31) infection, or mixed HPV-16 infections. *, negative for all paired or grouped probes targeting a specific nucleotide position(s); ?, a concurrent strong and weak hybridization signal at more than one of a pair or group of probes targeting a specified nucleotide position(s); #, a strong hybridization signal at more than one of a pair or group of probes targeting specified nucleotide position(s); $, only hybridization-based results, not DNA sequence-based information, are provided.

Fig. 2). These were the same two specimens identified as having mixed HPV-16 infections in the E6 studies. Although a single ambiguous overall L1 hybridization pattern was obtained for specimen 8392, two technical hybridization failures (6695A and 6994A) with this same specimen were observed. It should be noted that this specimen produced a small quantity of PCR product, as judged by EtBr staining following agarose gel electrophoresis. Of the 840 individual hybridizations to HPV-16-containing specimens (70 specimens with 12 probes), approximately 1% technical hybridization failures were observed in the L1 hybridization system described here.

All specimens containing HPV-16 and HPV-31 DNAs (n = 4) produced interpretable hybridization patterns except with the 6994 probe. In these specimens hybridization was strong with the HPV-16 reference probe (6994G) and weak with the 6994A variant probe. This result is explained by the HPV-31 nucleotide sequence in this region. Specimens containing only HPV-31 DNA (n = 3) failed to hybridize with all of the L1 probes except at nucleotide position 6994. At this nucleotide position, hybridization to the 6994A probe was observed.

The distribution of technical hybridization failures in specimens containing HPV-16 DNA alone was as follows: three 6695A probes, one 6721A probe, three 6854/62CT probes, and a single 6994A probe. Nucleotide sequence variations differed from the anticipated HPV-16 L1 hybridization pattern in 1 (<2%) of the 70 HPV-16-containing specimens. In this case an A at nucleotide position 6861 was observed in specimen OR.8863. No unanticipated linkage of individual E6 and L1 nucleotides or of overall hybridization patterns was observed. Representative E6 and L1 hybridization patterns and the corresponding sequences, when they were determined, are presented in Fig. 1 and 2.

DISCUSSION

To date, sequence diversity within HPV types and the significance of HPV variants have not been widely considered. This is largely due to the labor-intensive DNA sequencing efforts required to distinguish variant genomes. Identification of HPV-16 variant associations may prove important to the rational design of diagnostic, therapeutic, and vaccine strategies. In addition, because HPV type determinations have not

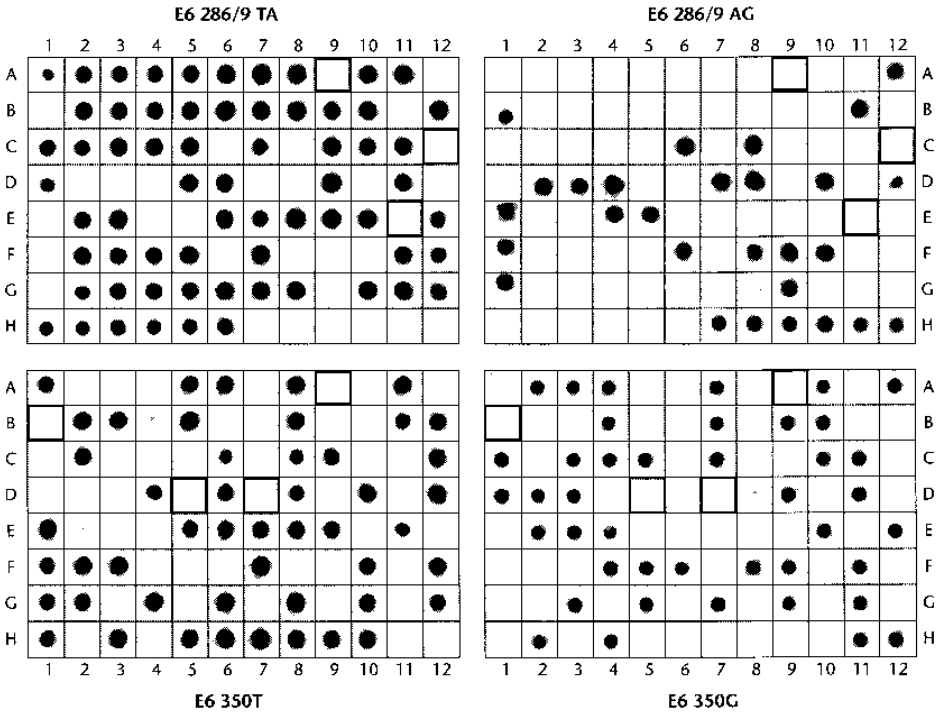


FIG. 3. Representative X-ray film exposures for E6-based hybridizations targeting nucleotides 350G, 350T, 286/9TA, and 286/9AG. Paired membranes 350G and 350T contain specimens different from those on the 286/9TA and 286/9AG membrane pair. Boxed wells designate specimens that were negative for all probes because of a lack of amplification. In row H, numbers 1 through 12 represent the control amplification reactions used to validate the specificity of the probe hybridization.

yet provided us with useful prognostic markers that are markedly better than cytologic screening, the role of intratype HPV variants in determining the risk for developing HPV-associated disease may become an active area of epidemiologic investigation. Recent studies have implicated variant HPV sequences as correlates of HPV-associated disease (12, 20, 35, 65, 68). The report of Ellis and coworkers (12) suggests that particular HPV-16 variants are associated with the HLA B-7 allele and the development of invasive cervical cancer.

We have previously identified a set of signature nucleotide patterns for each HPV-16 lineage that allows tentative classification of HPV-16 isolates (70). This catalog of signature nucleotides was used to develop PCR-based oligonucleotide probe systems capable of distinguishing all HPV-16 class and subclass variants reported in our earlier investigations.

Previous reports examining HPV-16 variants have targeted the LCR. These investigations were driven by the a priori hypothesis that genomic sequences usually diverge more in noncoding regions than in coding regions (34). However, our recent studies demonstrated that the HPV-16 E6-coding region identified distinct subclasses that were not identified by marker nucleotides within the LCR sequence (34). Like the LCR, E6 information can easily be obtained by targeting a short continuous segment of the HPV-16 genome. In addition, our previous studies demonstrated that nucleotide changes in one region of the HPV-16 genome can be used as markers of nucleotide changes in other regions within the same HPV-16 lineage. Using oligonucleotide probes that targeted these previously identified nucleotide changes, we found, as predicted, that nucleotide changes within the MY09/11 region of the L1-coding segment were linked to E6 nucleotide changes. Thus, through examination of the E6-coding region, L1 marker nucleotides and HPV-16 lineage characteristics in distant genomic segments can be inferred.

The hybridization methods presented here are capable of distinguishing single nucleotide changes at selected marker nucleotide positions within the amplified fragments targeted. We have determined that the chosen primer sequences are conserved between all HPV-16 variants previously reported by our group; thus, preferential amplification by one variant over another should not occur. In reconstruction experiments, an HPV-16 variant representing >10% of the input PCR product can be detected (data not shown). Therefore, coinfection with an HPV-16 variant present at a low copy number may go undetected by this method. The sensitivities of these assays could most certainly be augmented by applying a greater quantity of PCR product onto the hybridization membrane. In our study of selected specimens, we detected more than a single HPV-16 variant in less than 2% of individuals. Previous investigations have observed multiple HPV-16 variants in a significantly higher percentage of individuals (25, 67). The reason for this discordance with previously reported data is possibly explained by the differences in the populations examined and the methods used. In this study, we selected individuals who were predominantly positive for HPV-16 DNA alone (i.e., no other HPV types were detected) by consensus PCR and type-specific hybridization (18). The assays used in our study target distinct lineage marker nucleotides, whereas investigations that use single-strand conformational polymorphism or sequence analysis of cloned PCR products would more readily detect random nucleotide changes not characteristic of distinct HPV-16 lineages. Although these alternate methods are potentially capable of identifying unique HPV variants, the results obtained by these methods will also be more subject to confounding by *Taq* polymerase-induced errors. Subsequent studies from our group examining more than 1,000 individual HPV-16-positive specimens suggest that unique nonlineage nucleotide changes within E6, the MY09/11 region of L1, and the LCR are found

in a minority of HPV-16 variants and that these nucleotide changes are not observed in other isolates. The novel HPV-16 variants identified in these subsequent studies, including a worldwide collection of invasive cervical cancers (5, 69), represent less than 8% of all HPV-16 variants that we have encountered. Although the current probe systems detect more than 92% of the HPV-16 variant lineages reported to date, hybridization probes are under development to facilitate the identification of these additional HPV-16 variants.

It should be noted that in our subsequent investigations of randomly selected study subjects ($n = 300$), we also observed an approximate 1% prevalence of "mixed" HPV-16 variant infections (64). If mixed HPV-16 variant infections are, in fact, rare, this observation would potentially support a model for immunological protection by one HPV-16 variant infection from a subsequent HPV-16 infection. As discussed by other investigators, the establishment of a persistent infection in which a single HPV-16 variant predominates over time may similarly provide indirect support for such a model (59, 67). This suggestion contrasts with the finding that HPV infections are commonly observed as mixed infections with multiple HPV types (2, 3, 22, 39, 66). Although definitive studies have not been reported, there appears to be little immunologic cross protection provided by previous infections with multiple HPV types. It is curious that a spectrum of copy number ratios for groups or pairs of HPV-16 variants was not observed within the populations of individuals that we have examined. One would expect that although our results may be subject to methodologic bias, we should easily have detected distinct HPV-16 variants representing 25% or more of the total HPV-16 variants present. We are currently examining significant numbers of cloned HPV-16 molecules generated by PCR from single individuals using this hybridization method. These studies, accompanied by targeted DNA sequencing efforts, will help clarify our previous observations. Hybridization with HPV-16 lineage-specific oligonucleotide probes will allow for the rapid screening of multiple clones that may represent HPV-16 variants not detected initially when screening PCR product pools. Further investigations of HPV-16 variant infections and studies comparing methods for their detection are warranted, especially in the context of longitudinal natural history studies.

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